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Co-Existence of Inoculated Yeast and Lactic Acid Bacteria and Their Impact on the Aroma Profile and Sensory Traits of Tempranillo Red Wine

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Abstract: This study investigates the effects of simultaneous inoculation of a selected *Saccharomyces cerevisiae* yeast strain with two different commercial strains of wine bacteria *Oenococcus oeni* at the beginning of the alcoholic fermentation on the kinetics of malolactic fermentation (MLF), wine chemical composition, and organoleptic characteristics in comparison with spontaneous MLF in Tempranillo grape must from Castilla-La Mancha (Spain). Evolution of MLF was assessed by the periodic analysis of L-malic acid through the enzymatic method, and most common physiochemical parameters and sensory traits were evaluated using a standardized sensory analysis. The samples were analyzed by GC/MS in SCAN mode using a Trace GC gas chromatograph and a DSQII quadrupole mass analyzer. Co-inoculation reduced the overall fermentation time by up to 2 weeks leading to a lower increase in volatile acidity. The fermentation-derived wine volatiles profile was distinct between the co-inoculated wines and spontaneous MLF and was influenced by the selected wine bacteria used in co-inoculation. Co-inoculation allows MLF to develop under reductive conditions and results in wines with very few lactic and buttery flavors, which is related to the impact of specific compounds like 2,3-butanedione. This compound has been also confirmed as being dependent on the wine bacteria used.

Keywords: Simultaneous inoculation; Alcoholic fermentation; Malolactic fermentation; *Sacccharomyces cerevisiae*; *Oenococcus oeni*; PN4^{TM}; Omega^{TM}; Aroma profile

1. Introduction

Malolactic fermentation (MLF) occurs in wine as the result of the metabolic activity of wine lactic acid bacteria. It can take place after the alcoholic fermentation, normally carried out by yeasts, but it can occur earlier. MLF reduces wine acidity by the conversion of dicarboxylic L-malic acid (malate) into monocarboxylic L-lactic acid (lactate) and carbon dioxide, which implies a modification in wine flavor and its microbiological stability, both of which are considered to be beneficial effects for wine quality [1,2]. Sensory studies carried out in the 1980s by Davis et al. [3] showed that malolactic activity of bacteria imparts recognizable changes to the flavor characteristics of wine. These modifications come from the biotransformation and enzymatic processing of grape nutrients into flavor-active compounds [4].

Several studies show that the combination of selected wine yeasts and bacteria strains has different sensory effects in wines due to the production of certain impact metabolites. They are synthesized in different amounts by these organisms according to the grape variety and bacterial strain, giving us the opportunity to determine if their contribution to aroma and flavor depends on the species or strains [5–7]. Research performed by Pozo-Bayón et al. revealed different malolactic behavior for *Oenococcus oeni* and *Lactobacillus plantarum*, concluding that MLF by both species may contribute to wine quality by modifying the concentration of some of the amino acids and aroma compounds of wine because of metabolic differences [8]. However, according to other previous studies, it was proved that the influence of lactic acid bacteria on wine sensory attributes is strain specific. Experiments carried out using different strains of *O. oeni* showed significant changes in aroma and flavor compounds, such as the modifications in fruity characters in red wines or the greater complexity acquired in Sauvignon Blanc wines [9–11].

Lactic acid bacteria can also produce undesirable aroma and flavor compounds, such as volatile phenols or biogenic amines, both related with quality loss and health problems for humans [12,13]. This problem could be solved with a custom selection of bacterial strains, according to the type of wine and its organoleptic properties, which would be modulated depending on the searched sensory profile and consumer preferences [10,14]. Additionally, the use of selected strains of wine bacteria allows for better control of the timeframe of L-malic acid degradation [15,16].

Once the wine bacterial strains are selected, another relevant parameter to study is the timing of the bacteria starter addition and the number of cells and viability in the wine after inoculation, since these can influence the sensory profile of wine. Three different strategies are being investigated: (i) inoculation at any time during alcoholic fermentation often performed simultaneously with yeast (co-inoculation); (ii) inoculation at pressing; and (iii) inoculation after the alcoholic fermentation (sequential inoculation) [17]. Co-inoculation of yeast and bacteria during fermentation shows several benefits when compared to the other strategies, as it shortens vinification times, enhances the wine organoleptic characteristics and reduces the probability of microbial spoilage [2]. Co-inoculation also influences the volatile chemical composition and the potential sensory attributes of the wine, often giving fruitier notes, as opposed to butter or nuts when the MLF begins after the end of the alcoholic fermentation [18].

The aim of the present study is to determine the effect of the coexistence of inoculated commercial yeast and novel selected lactic acid bacteria strains and its impact on the aroma profile and sensory traits of Tempranillo red wine, by analyzing chemical, physical, and sensory parameters.

2. Materials and Methods

2.1. Microorganisms

The yeast strain selected to perform alcoholic fermentation was Viacell C-58® (Lallemand Inc., Montreal, Quebec, Canada). This *Saccharomyces cerevisiae* yeast strain was isolated in Germany and used according to the manufacturer's recommendations. It has the ability to release polysaccharides to the wine, stabilizing color in red wines, and decreasing astringency of green tannins. Furthermore, it stimulates the development of fruity aromas that persist after barrel aging. This yeast strain also shows a high adaptation to low temperatures, which makes it suitable for cold pre-fermentative maceration.

The commercial wine lactic acid bacteria (LAB) used were *O. oeni* PN4™ (Lallemand Inc., Montreal, Quebec, Canada) and *O. oeni* Omega™ (Lallemand Inc., Montreal, Quebec, Canada). LAB strain PN4™ was isolated and selected at the Edmund Mach Institute (San Michele all'Adige, Trentino, Italy). This LAB stands out for being a robust strain, which showed the ability to complete malolactic fermentation in red and white wines under adverse conditions of pH, alcohol, SO_2, and temperature. In red wines, PN4™ seems to contribute to spicy notes. LAB strain *O. oeni* Omega™ was isolated and selected in the Languedoc region in France by the Institut Française de la Vigne et du Vin (IFV). According to the previous studies made by the manufacturer, it stands out for its ability to finish

rapidly malolactic fermentation in a wide range of applications. This LAB endures low pH and high alcohol levels. It is effective in red, rose, and white wines. It complements a fresh and direct fruit style and helps to stabilize color due to its slow degradation of acetaldehyde. Both LAB had been described by the manufacturer (Lallemand Inc., Montreal, Quebec, Canada) to be highly suitable for the use as components of a mixed yeasts/bacteria co-inoculum.

2.2. Vinifications

Tempranillo grape-berries were harvested at the optimum technological maturity (23–25 °Brix) during the 2018 vintage in the Castilla-La Mancha winemaking area. Once grapes were received in the cellar, they were crushed and treated with 4 g/hL of SO_2, and then a dose of 2 g/hL of IOC enzyme (Lallemand Inc., Montreal, Quebec, Canada) was added to favor the color extraction. The initial chemical composition of must was: °Brix 23.50; total acidity 5.35 g/L; pH 3.85, and L-malic acid 1.95 g/L. Must with crushed grapes was homogeneously divided after crushing into six stainless steel vessels with a capacity of 100 L. Two of the vessels were used as control (AF1), in which AF means Alcoholic Fermentation and no lactic acid bacteria strains were added, and after alcoholic fermentation with the selected *S. cerevisiae*, wines carried out malolactic fermentation spontaneously. The four remaining deposits were inoculated with commercial *O. oeni* lactic acid bacteria strains 24 h after yeast inoculation, two of them with PN4^{TM} (AF2) and the other two with Omega^{TM} (AF3). The obtained wines were cold-stabilized at –3 °C for 25 days, and afterwards they were filtered and bottled. Once they were bottled, wines were sensorily and chemically analyzed.

2.3. Microbiological Control

Determination of microbial population was carried out as stated by Izquierdo-Cañas et al. [16]. The viable count of yeasts was performed by plating samples in malt extract agar media (Cultimed, Barcelona, Spain), and incubated at 28 °C for 48 h. In order to quantify lactic acid bacteria, samples were plated onto MLO Agar (MLOA, *O. oeni* Medium, Scharlab, Barcelona, Spain) supplemented with 10% *v/v* tomato juice, 50 mg/L sodium azide, and 100 mg/mL of cycloheximide. Plates were incubated under anaerobic conditions (Gas Pack System, Oxoid Ltd, Basingstoke, UK) at 30 °C for 5 days. The results were expressed in colony forming units per mL (CFU/mL) of wine.

The implantation of the inoculated lactic acid bacteria was assessed by the random selection of isolated colonies from MLOA plates at different fermentation times after inoculation, when L-malic acid concentration had decreased to 50% and when L-malic acid content was below 0.2 g/L.

2.4. FML Progress and Physicochemical and Spectrophotometric Analysis

FML evolution was determined by the periodic analysis of L-malic acid content, using the enzymatic method established by the International Organization of Vine and Wine (OIV) [19]. Most common physiochemical parameters, such as alcoholic strength, pH, total acidity, total and free SO_2, glucose and fructose content, and Folin–Ciocalteu index to determine the total phenolic content of wines were also measured following the recommendations of the official methods of the OIV [20]. Colorimetric parameters were measured at pH 3.6 by a CIE-Lab* color system following the protocol proposed by Ayala et al. [21]. Total anthocyanin content was measured by the sulfur dioxide bleaching method according to Riberèau-Gayon and Stonestree [22]. Concentration of different carboxylic acids (malic, lactic and citric acid) and glycerol content were determined by an isocratic HPLC system that was set up with a column block heater and refractive index detector. The mobile phase was 8 mmol/L H_2SO_4, 0.6 mL/min, and set at 75 °C in an Aminex HPX-87H 300 × 7.8 mm column. Finally, tannin content in wines was quantified by precipitation with methylcellulose following the protocol proposed by Sarneckis et al. [23].

2.5. Volatile Compound Analysis

Volatile compound extraction was performed according to the protocol described by Ibarz et al. [24]. SPE cartridges (Li-Chrolut EN, Merck, 0.2 g of phase, Darmstadt, Germany) and 4-nonanol (0.1 g/L) were used as internal standards. The extracts obtained were concentrated to a final volume of 150 µL by distillation in a Vigreux column and then under a nitrogen stream. Samples were kept at –20 °C until further analysis. A Focus-GC system coupled to a mass spectrometer ISQ with electron impact-ionization source and quadrupole analyzer with an auto-sampler Tri-Plus (Thermo-Quest, Waltham, MA, USA) was used to assess the free volatile composition of wines. We used a BP21 column (SGE, Ringwood, Australia) with a 50 m × 0.32 mm internal diameter and 0.25 µm thick of Free Fatty Acid Phase (FFAP). The chromatographic conditions were as follows: carrier helium gas (1.4 mL min^{-1}, split 1/57); injector temperature, 220 °C; and oven temperature, 40 °C for 15 min, 2 °C min^{-1} to 100 °C, 1 °C/min to 150 °C, 4 °C/min to 210 °C, and 55 min at 210 °C. The detector conditions were as follows: impact energy, 70 eV; electron multiplier voltage, 1250 V; ion source temperature, 250 °C; and mass scanning range, 40–250 amu. Volatile compounds were detected by chromatographic retention times and mass spectra, using commercial products as standards. Quantification of volatile compounds was carried out by analyzing the characteristic m/z fragment for each compound using the internal standard method. Concentrations of non-available volatile compounds were expressed at µg/L or mg/L as 4-nonanol equivalents obtained by normalizing the peak area to that of the internal standard and multiplying by the concentration of the internal standard.

2.6. Sensory Analysis

Descriptive and triangular tests were first performed to determine the differences among treatments. Fifteen tasters were selected to carry out a descriptive sensory analysis according to the Sensory Profile method, based on a scale from 0 (absence of the descriptor) to 5 (maximum intensity), and established by the ISO standard [25]. Selected attributes were color intensity, purplish-red color, aroma of red fruits, aroma of ripe red fruits, aroma of raisins, floral bouquet, aroma of spices, milky aromas, fresh aroma, astringent flavor, noble mature tannins in the mouth, and lengthy finish in the mouth.

2.7. Statistical Analysis

Significant differences among samples were determined for each chemical compound by analysis of variance (ANOVA) and paired Student's t-test ($p = 0.05$) for each chemical compound. A cobweb graph was performed to determine significant differences of the attributes after sensory analysis. Statistical analysis was performed with 20.0 SPSS (IBM Inc. Chicago, IL, USA) for Windows statistical package.

3. Results

3.1. Evolution of Microbial Populations and Fermentation Kinetics

The progress of alcoholic fermentation of the commercial *S. cerevisiae* Viacell C-58® strain was evaluated for each vessel by daily density measurement under the established experimental conditions: spontaneous malolactic fermentation, co-inoculation with *O. oeni* PN4™, and co-inoculation with *O. oeni* Omega™. After seven days, since density was always below 993 g/L, alcoholic fermentation was considered to be finished with similar results in all wines (Figure 1).

Although the presence of native LABs could have an adverse effect in non-sterile must fermentation, our results showed the favorable effect on the start-up period of inoculating pure LAB cultures. The presence of native lactic acid bacteria in the first stages of alcoholic fermentation did not affect the progressive decrease of density in wine (Figure 1). In fact, in the spontaneous malolactic fermentation (control), it was observed that population of *S. cerevisiae* was affected by the presence of native lactic acid bacteria, maintaining an average concentration of 10^6 cells/mL when must density decreased, until LAB took part in malolactic fermentation (Figure 1). In the co-inoculated vinification with the *O. oeni* PN4™ strain, the development of the bacterial population showed a good adaptation to

fermentation conditions, following fermentative kinetics similar to the corresponding kinetics of Viacell C-58® yeast. Conversely, in the co-inoculated fermentation with Omega™, a different behavior was observed in presence of Viacell C-58® during the first days of the alcoholic fermentation, reaching concentrations between 10^7 and 10^8 cells/mL, while it reached similar populations to PN4™ during the rest of the alcoholic fermentation (Figure 1).

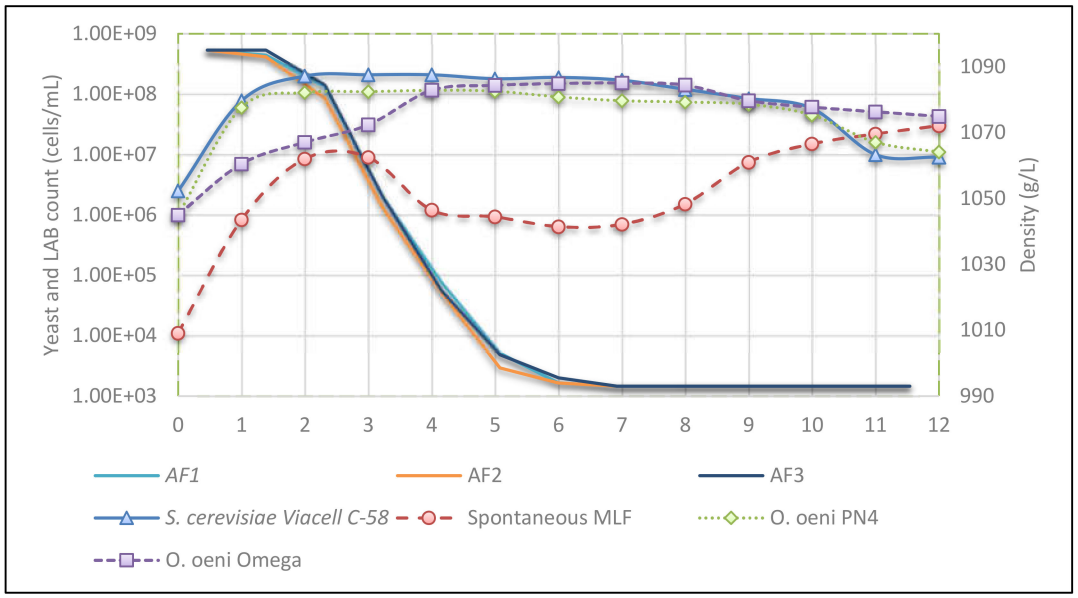

Figure 1. Progress of the yeast Viacell C-58® (**Δ**) and lactic acid bacteria population (dashed lines) and must density (continuous lines) during the three wine alcoholic fermentations denoted as (AF1, AF2, and AF3) and performed with Tempranillo musts using the following inoculation strategies: no lactic acid bacteria (LAB) inoculation (•); inoculation with LAB PN4™ (♦); and inoculation with LAB Omega™ (■). Data represented correspond to mean values obtained from duplicate fermentations; in all cases standard deviation was <1.5. MLF means malolactic fermentation.

3.2. Malolactic Fermentation

Malolactic fermentation dynamics were controlled by the daily determination of L-malic and L-lactic acid content. In co-inoculated fermentation with PN4™ and Omega™ strains, the behavior was similar during the first days, but variations in L-malic acid consumption appeared in the final stages of the alcoholic fermentation (Figure 2). The strain PN4™ could completely consume L-malic acid in 10 days in the presence of *S. cerevisiae* starters, while the Omega™ strain did the same two days later. Regarding the spontaneous malolactic fermentation, the consumption of L-malic acid was started from day 12, finishing MLF in 26 days (Figure 2). Again, this fact confirms that lactic acid bacteria co-existed with *S. cerevisiae* Viacell C-58® until L-malic acid was almost finished. It is important to highlight that the L-malic acid consumption was reduced between 14 and 16 days when *O. oeni* PN4™ and Omega™ strains were inoculated compared to the spontaneous MLF.

3.3. Determination of Physic-Chemical Parameters of Fermentations and Color

Despite observing significant differences when comparing residual sugars in both co-inoculated and control vinifications, in all cases must sugars were completely consumed, and dry wines were obtained. Regarding acetic acid levels, they decreased to 0.28 g/L for PN4™ and 0.26 g/L for Omega™ values, which were lower than those obtained for spontaneous MLF wine, 0.35 g/L (Table 1).

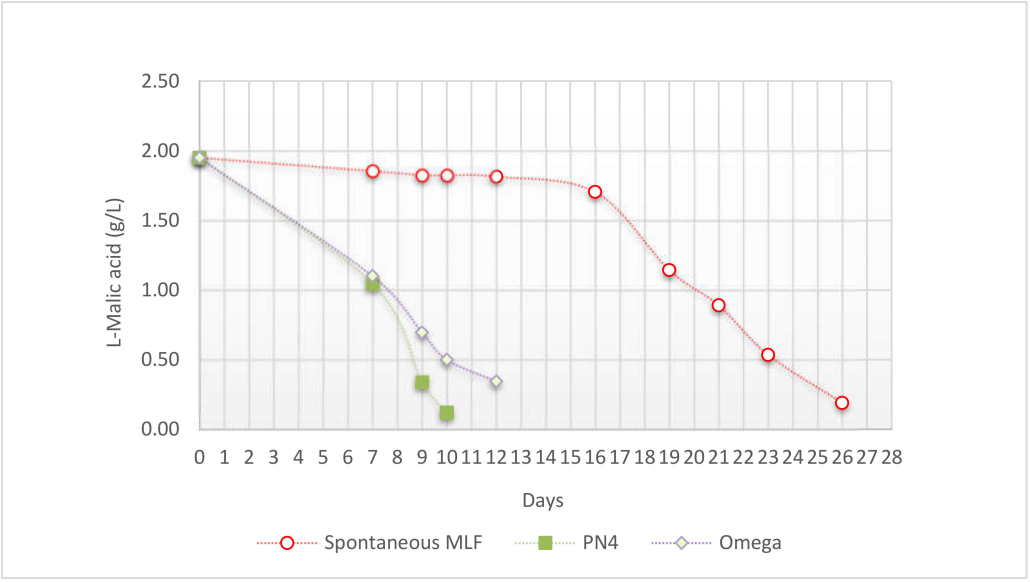

Figure 2. Progression of L-malic acid consumption (g/L) during vinification of Tempranillo must in the two samples inoculated with LAB PN4^{TM} (♦) and with LAB Omega^{TM} (■) at the beginning of the alcoholic fermentation and the uninoculated one (●). Data represented correspond to mean values obtained from duplicate fermentations; in all cases standard deviation was <1.5.

Table 1. Physicochemical parameters, organic acid concentrations, and color values of wines. Data represented correspond to mean and standard deviation values obtained from duplicate vinifications. Characters a, b, and c mean significant differences at $p < 0.05$.

	Spontaneous MLF	**PN4^{TM}**	**Omega^{TM}**
Alcoholic strength (% v/v)	13.34 ± 0.66 ^{a}	13.45 ± 0.15 ^{a}	13.52 ± 0.23 ^{a}
Total acidity (g/L)	3.49 ± 0.03 ^{a}	3.62 ± 0.18 ^{a}	3.63 ± 0.18 ^{a}
pH	4.07 ± 0.08 ^{a}	4.04 ± 0.07 ^{a}	4.01 ± 0.03 ^{a}
Volatile acidity (g/L)	0.35 ± 0.01 ^{b}	0.28 ± 0.03 ^{a}	0.26 ± 0.00 ^{a}
L-malic acid (g/L)	0.07 ± 0.04 ^{a}	0.05 ± 0.01 ^{a}	0.08 ± 0.02 ^{a}
L-lactic acid (g/L)	1.27 ± 0.00 ^{c}	1.22 ± 0.02 ^{b}	1.12 ± 0.06 ^{a}
Citric acid (g/L)	0.07 ± 0.03 ^{a}	0.20 ± 0.04 ^{b}	0.19 ± 0.04 ^{b}
Glycerol (g/L)	9.69 ± 0.93 ^{a}	9.91 ± 0.26 ^{a}	9.85 ± 0.28 ^{a}
L*	17.13 ± 3.29 ^{a}	15.34 ± 0.36 ^{a}	19.26 ± 2.23 ^{a}
a*	43.39 ± 1.31 ^{a}	43.46 ± 0.68 ^{a}	44.38 ± 0.09 ^{a}
b*	22.13 ± 0.84 ^{a}	21.28 ± 0.18 ^{a}	20.99 ± 0.82 ^{a}
Color intensity	6.44 ± 0.83 ^{a}	6.79 ± 0.32 ^{a}	5.89 ± 0.55 ^{a}
Color tone	0.83 ± 0.00 ^{b}	0.76 ± 0.04 ^{a}	0.79 ± 0.04 ^{ab}
Total anthocyanins (mg/L)	446.00 ± 39.60 ^{a}	432.00 ± 33.94 ^{a}	426.50 ± 27.58 ^{a}
Catechins (mg/L)	694.05 ± 67.39 ^{a}	730.10 ± 107.48 ^{a}	614.60 ± 55.58 ^{a}
Tannins (mg/L)	0.61 ± 0.12 ^{a}	0.64 ± 0.15 ^{a}	0.51 ± 0.30 ^{a}
Folin–Ciocalteu	33.00 ± 4.24 ^{a}	34.50 ± 3.54 ^{a}	31.00 ± 1.41 ^{a}

There were no significant differences in volatile acidity concentrations between the two co-inoculated wines, while the spontaneous MLF showed a higher volatile acidity (Table 1). Total acidity, glycerol, and pH were similar in all cases, which indicates that co-inoculation with commercial lactic

acid bacteria did not affect in a negative way the chemical properties of wine (Table 1). L-lactic acid values differed for the three experimental conditions, and co-inoculated wine with the LAB Omega^{TM} showed the lowest values and the spontaneous MLF the highest. On the other hand, a decrease in citric acid content was observed in the spontaneous malolactic fermentation (Table 1). This fact indicates that citric acid degradation depends on the LAB strain used. In this study, the commercial strains degraded less citric acid and, as a consequence, the volatile acidity produced was lower than that of the non-co-inoculated one (Table 1). It is well known that during MLF, wine discoloration can take place. However, in this work, it was proved that there were no important differences in the spectral characteristics of wine color, and intensity was similar in all cases. Co-inoculation did not decrease wine color. No significant differences were observed in the rest of analyzed parameters (Table 1).

3.4. Volatile Compound Analysis

Aromatic profiles from the three different wines are shown in Table 2. Metabolism from Viacell C-58® and the native and commercial lactic acid bacteria decisively influenced the volatile composition of wine, including secondary aromas related to MLF. It is appreciated that esters and alcohols were significantly higher, and fatty acid content was lower in wines made by co-inoculation with commercial LAB strains compares to that made by spontaneous MLF, used as a control.

Ethyl acetate content was significantly different between the three fermentation strategies (Table 2). Fermentations carried out with the *O. oeni* Omega^{TM} strain showed a higher isoamyl acetate and hexyl acetate content, which are remarkable compounds in Tempranillo wines when compared to the other two wines. The lower content of 2-phenyl acetate was lower in the wine co-inoculated with the *O. oeni* PN4^{TM} strain (Table 2). Regarding the ethyl lactate, the LAB strain Omega^{TM} contributed to lower levels of lactic aromas, while the isoamyl acetate concentration was higher (Table 2).

Ethyl hexanoate is responsible for the fruity notes in red wines, and it was observed that its content was significantly higher in those wines made with *O. oeni* PN4^{TM} and Omega^{TM}, if compared to spontaneous MLF (Table 2). The same happened with 1-hexanol, which contributed to herbaceous notes (Table 2). It should also be emphasized that ethyl butyrate was significantly higher in wines made with Omega^{TM} (Table 2). No significant differences in the ethyl octanoate and ethyl decanoate contents were found between the three wine trials (Table 2).

Carboxylic acids are responsible for fatty, rancid, and buttery notes (Table 2). It was shown that co-inoculated wines had lower levels of hexanoic acid, octanoic acid, and decanoic acid with respect to the spontaneous MLF, highlighting that *O. oeni* PN4^{TM} notably reduced butyric acid and hexanoic acid content.

Taking into account that acetaldehyde content decreased during MLF, a decline was observed in wines with spontaneous MLF, in contrast with LAB co-inoculated wines, where PN4^{TM} showed the highest level of the compound. The 2,3-butanodione is a decisive compound in MLF, since it is mainly derived from lactic acid bacteria metabolism. High concentrations of this metabolite give heavy milky notes. Therefore, it is interesting to obtain it in low concentrations, as it will contribute to wine smoothness. High quantities of this compound were produced in spontaneous MLF wine in contrast to the co-inoculated ones. This fact can be very promising for wines produced by co-inoculation of commercial LAB since they have less lactic notes (octanoic and decanoic acid, 2,3-butanodione), which avoids the masking of fruity notes that give personality to Tempranillo wines made with the co-inoculation of *O. oeni* PN4^{TM} and Omega^{TM} strains.

3.5. Sensory Analysis

Regarding the sensory profile of wines and according to the tasters, differences in organoleptic properties were observed (Figure 3). Wines made by spontaneous MLF showed higher aromas of raisins, milky aromas, and color intensity. In contrast, wines developed by the inoculation of commercial lactic acid bacteria highlighted the fresh aroma and lengthy finish flavor and ripe red fruit character and less milky aromas (Figure 3).

Table 2. Data (Mean ± S.D.) of volatile composition related to the uninoculated fermentations (S) and inoculated fermentations with two commercial LAB strains (PN4™ and Omega™). Characters a, b, and c mean significant differences at $p \leq 0.05$.

	Spontaneous MLF	**PN4™**	**Omega™**
Ethyl acetate (mg/L)	80.00 ± 15.89 ^{a}	95.18 ± 0.13 ^{b}	108.65 ± 2.93 ^{c}
Isoamyl acetate (mg/L)	5.85 ± 0.76 ^{a}	6.13 ± 0.10 ^{a}	8.85 ± 0.89 ^{b}
2-Phenylethyl acetate (μg/L)	182.61 ± 7.24 ^{b}	141.89 ± 7.89 ^{a}	170.63 ± 31.80 ^{b}
Hexyl acetate (μg/L)	13.54 ± 1.77 ^{a}	13.75 ± 0.84 ^{a}	21.21 ± 7.18 ^{b}
Ethyl lactate (mg/L)	145.86 ± 19.96 ^{b}	137.78 ± 6.11 ^{b}	117.54 ± 1.24 ^{a}
Ethyl butyrate (μg/L)	632.50 ± 106.77 ^{a}	687.50 ± 30.41 ^{a}	896.00 ± 94.75 ^{b}
Ethyl hexanoate (μg/L)	739.82 ± 26.96 ^{a}	790.88 ± 57.99 ^{ab}	866.78 ± 85.64 ^{b}
Ethyl octanoate (μg/L)	716.64 ± 49.17 ^{a}	677.01 ± 90.65 ^{a}	785.66 ± 85.41 ^{a}
Ethyl decanoate (μg/L)	97.26 ± 8.89 ^{a}	90.29 ± 14.61 ^{a}	100.97 ± 10.01 ^{a}
1-propanol (mg/L)	62.83 ± 8.25 ^{a}	54.74 ± 5.25 ^{a}	54.55 ± 0.51 ^{a}
Isobutanol (mg/L)	30.48 ± 3.71 ^{a}	28.96 ± 0.51 ^{a}	30.65 ± 1.20 ^{a}
1-butanol (mg/L)	1.46 ± 0.30 ^{a}	1.65 ± 0.12 ^{a}	1.46 ± 0.23 ^{a}
Isoamylic alcohols (mg/L)	342.50 ± 36.63 ^{a}	332.19 ± 1.25 ^{a}	327.92 ± 10.87 ^{a}
1-octanol (μg/L)	13.82 ± 2.45 ^{a}	16.14 ± 2.27 ^{a}	17.33 ± 1.77 ^{a}
3-methyl-1-pentanol (μg/L)	167.16 ± 42.26 ^{a}	212.66 ± 6.96 ^{c}	181.06 ± 1.16 ^{ab}
3-ethoxy-1-propanol (μg/L)	53.47 ± 13.10 ^{a}	48.15 ± 3.02 ^{a}	45.46 ± 2.95 ^{a}
1-hexanol (mg/L)	3.24 ± 0.12 ^{a}	3.46 ± 0.16 ^{b}	3.53 ± 0.08 ^{b}
t-3-hexenol (μg/L)	74.70 ± 0.47 ^{a}	75.24 ± 9.86 ^{a}	74.74 ± 0.86 ^{a}
c-3-hexen-1-ol (μg/L)	513.62 ± 88.94	516.16 ± 21.29	560.66 ± 4.26
2-phenylethanol (mg/L)	23.68 ± 2.79 ^{a}	26.07 ± 1.58 ^{a}	22.54 ± 1.49 ^{a}
Isobutiric acid (μg/L)	460.53 ± 49.70 ^{a}	428.63 ± 51.80 ^{a}	426.93 ± 10.52 ^{a}
Butyric acid (μg/L)	570.86 ± 1.69 ^{b}	436.89 ± 2.96 ^{a}	513.28 ± 91.90 ^{b}
Isovaleric acid (mg/L)	1.43 ± 0.05 ^{c}	1.31 ± 0.08 ^{b}	1.22 ± 0.06 ^{a}
Valeric acid (μg/L)	13.68 ± 3.89 ^{b}	7.71 ± 2.71 ^{a}	6.68 ± 4.72 ^{a}
Hexanoic acid (mg/L)	8.29 ± 0.46 ^{b}	7.28 ± 0.57 ^{a}	7.80 ± 0.34 ^{ab}
Octanoic acid (mg/L)	7.25 ± 1.65 ^{b}	5.02 ± 0.08 ^{a}	5.41 ± 0.16 ^{a}
Decanoic acid (mg/L)	3.78 ± 0.94 ^{b}	2.08 ± 0.10 ^{a}	1.92 ± 0.17 ^{a}
γ-nonalactone (μg/L)	6.35 ± 0.53 ^{a}	5.39 ± 0.56 ^{a}	6.14 ± 0.55 ^{a}
δ-dodecalactone (μg/L)	9.58 ± 0.67 ^{a}	6.89 ± 1.05 ^{a}	7.56 ± 3.70 ^{a}
δ-ethoxycarbonyl-δ-butyrolactone (μg/L)	321.46 ± 39.16 ^{ab}	275.65 ± 26.25 ^{a}	355.95 ± 31.85 ^{b}
Damascenone (μg/L)	6.03 ± 0.81 ^{a}	6.27 ± 0.23 ^{a}	5.74 ± 0.97 ^{a}
3-oxo-α-ionol (μg/L)	55.82 ± 0.60 ^{a}	49.64 ± 7.01 ^{a}	53.82 ± 4.76 ^{a}
Acetaldehyde (mg/L)	9.28 ± 0.05 ^{a}	10.87 ± 0.96 ^{b}	14.00 ± 1.16 ^{c}
2.3 Butanodione (mg/L)	18.35 ± 1.67 ^{c}	14.77 ± 1.61 ^{b}	8.55 ± 0.48 ^{a}
1-octen-3-ona (μg/L)	14.25 ± 0.59 ^{a}	14.02 ± 1.84 ^{a}	14.19 ± 2.08 ^{a}
3-methyl-thio-propanol (μg/L)	277.33 ± 85.07 ^{a}	311.44 ± 66.18 ^{a}	211.11 ± 10.75 ^{a}
Furaneol (μg/L)	12.32 ± 1.26 ^{b}	5.94 ± 3.73 ^{a}	10.39 ± 2.52 ^{b}
Guaiacol (μg/L)	12.39 ± 1.65 ^{a}	18.44 ± 0.37 ^{b}	19.88 ± 5.18 ^{b}

Table 2. *Cont.*

	Spontaneous MLF	PN4™	Omega™
Eugenol (µg/L)	3.97 ± 0.36 [b]	2.52 ± 0.79 [a]	2.71 ± 0.13 [a]
Phenol (µg/L)	8.33 ± 0.98 [a]	9.41 ± 0.66 [a]	10.24 ± 3.49 [a]
4-metil-2, 6-ditercbutil-fenol (µg/L)	95.91 ± 29.03 [b]	66.28 ± 8.35 [a]	63.06 ± 0.07 [a]

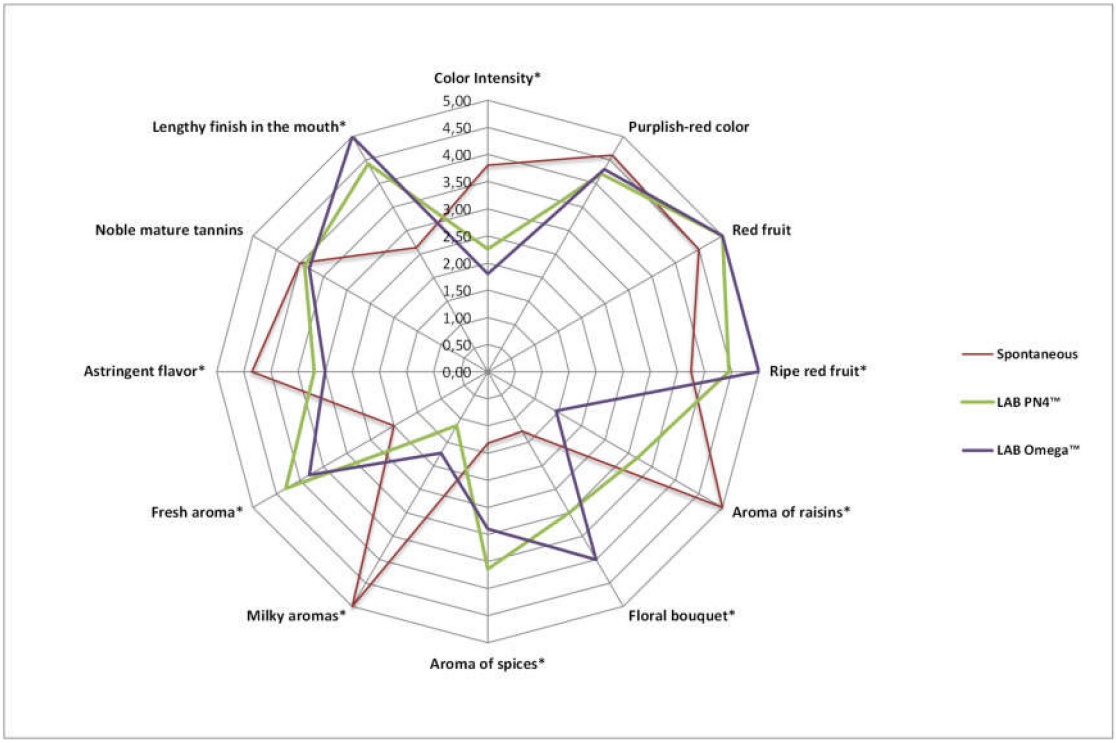

Figure 3. This figure shows the polar coordinate (cobweb) graph of mean sensory score ratings of "color intensity", "purplish-red color", "red fruit", "ripe red fruit", "aroma of raisins", "floral bouquet", "aroma of spices", "milky aroma", "fresh aroma", "astringent flavor", "noble mature tannins", and "lengthy finish in the mouth" for the spontaneous Tempranillo wine and the PN4™ and Omega™ LAB strains. In sensorial variables indicated with an asterisk (*) a difference between trials was verified for $p \leq 0.05$.

Panelists concluded that wines fermented with LAB reached more fruity and floral flavors, and spontaneous wines were less fruity and had stronger astringency and milky, raisin flavors. LAB Omega™ had strongly fruity and floral aromas, and PN4 in spices and fresh aromas in the Tempranillo wines. Spontaneous LAB produced the least ripe red fruit, floral, spices, and fresh aromas wines in this comparison. These wines tended to have milky, raisin, and astringent flavors. Statistical analysis revealed that the descriptors that the descriptors differed significantly in non-inoculated Tempranillo wines from those ones, which were inoculated,) suggesting that the different LAB strains deeply affected the flavor compounds of the wines (Figure 3).

4. Discussion

Under normal conditions, spontaneous malolactic fermentation (MLF) takes place once alcoholic fermentation has finished. This fact is due to a competition for must nutrients between yeast and lactic acid bacteria (LAB) naturally occurring in grapes. *S. cerevisiae* develops rapidly and consumes the essential nutrients for lactic acid bacteria growth, in addition to the production of ethanol and other byproducts that avoid their proliferation in the first stages of fermentation [26,27]. However,

as alcoholic fermentation finishes, yeast suffer what is called lysis or autolysis, where they free intracellular nutrients that will be used as nitrogen and carbon sources to those lactic acid bacteria that resisted the ethanol concentration of the final wine [27]. Then, lactic acid bacteria population will exponentially proliferate to carry out MLF. It is possible to reproduce this natural phenomenon in controlled conditions. When winemakers use selected or commercial yeast starters to perform alcoholic fermentation, generally they inoculate lactic acid bacteria in sequential steps reproducing the succession that would take place naturally.

Other winemakers decide to co-inoculate LAB with fermentative yeast at the beginning of fermentation or just before alcoholic fermentation finishes [27–29]. This technique is gaining popularity because not only MLF is assured, but also carries recognized advantages by enologists and winemakers. One of the most important benefits of simultaneous inoculation of yeast/LAB is the reduction of total time of fermentation [18]. Jussier et al. [30] observed a significant reduction of MLF time in Chardonnay wines at pH of 3.53 and 13% ethanol (*v*/*v*) in co-inoculation when compared to sequential AF/MLF. This study corroborates this assertion at a pilot scale and is consistent with previous investigations performed at the laboratory scale [29,31–34].

Reduction of MLF times can be explained by specific interactions that occur between S. *cerevisiae* and O. *oeni* during AF and MLF, when the chosen strategy is the co-inoculation of both starter cultures [35]. In the experiments of this study, Viacell C-58® was used as fermentative yeast and two *O. oeni* (PN4 ™ and Omega™) as commercial LAB as a mixed co-inoculum of yeast/bacteria. Yeast and LAB strains had been described by the producers to be highly suitable for the use as component of a mixed yeast/bacteria co-inoculum. It can be observed that interactions between the yeast and the commercial co-inoculated bacteria are different from those that take place in spontaneous fermentation. The concentration of the inoculated yeast was about 10^6 CFU/mL in all cases for the commercial LAB, while the LAB initial population of spontaneous elaborations was 10^4 CFU/mL, a little higher than that reported in red wine musts [36]. MLF also occurred when AF was finished, but the duration of the total process was substantially superior than in the case of the two starter culture inoculations, due to the adaptation of bacterial starter to the must environment from the beginning of AF. Furthermore, viability of S. *cerevisiae* starter did not get influenced during the simultaneous progress of AF and MLF, as the yeast exponential growth phase did not decrease before reaching the stationary phase [32–37]. These evidences are consistent with those obtained in previous studies in Cabernet Franc, Tempranillo, and Merlot wines with other LAB and yeast strains [16,38] and Neroamaro wines [29].

This study also confirmed that MLF can take place in the presence of fermentable sugars without a significant increasing of acetic acid, in contrast with data obtained in other studies where the content of acetic acid increased in yeast and LAB co-inoculations [12,39,40]. This variability in the reduction of volatile acidity in co-inoculated wines with PN4™ and Omega™ strains could depend on the yeast strain used, on the LAB starter used, or both. This result shows consistency with those obtained by Tristezza et al. [29] in Neroamaro wines and du Plessis et al. [41] in Shiraz wines. In fact, in the vinification experiments, approximately 50% of the malic acid was consumed at the 7th day of the AF, and the total consumption of malic acid took place after the AF (days 7–12). This result reconfirmed a previous study of this research group in which an improvement of organoleptic properties of Cabernet Franc, Merlot, and Tempranillo wines was observed [16,38].

Regarding the color, no significant differences were observed in the spectral properties of color in co-inoculated MLF when compared to the control, but in contrast, lower shades were found in LAB co-inoculated wines. According to Burns and Osborne [42], MLF can affect red wine color independently of pH change. The species *O. oeni* could change the concentration of phenolic and non-phenolic compounds involved in red wine color development independently of pH change. As stated before, there were no strain specific differences in the color, but the tonality could be related to the *O. oeni* strain used.

The obtained results not only confirm that the correct selection of yeast and LAB strains for the performing of MLF by co-inoculation has a positive influence in the reduction of fermentation times

and volatile acidity production, but also in the aromatic composition of wine. In fact, it was clearly proved the considerable effect of co-inoculation of the commercial yeast with two different LAB strains on the organoleptic properties of Tempranillo wines obtained, when compared with those obtained by spontaneous MLF. Recent investigations have highlighted the variation in aromatic profile in wine produced by different LAB inoculation processes [16,18,38,39]. Our data suggest that coexistence of yeast *Saccharomyces* with the lactic acid bacterial strain Omega^{TM} increases some ester levels, improving the fruity aroma according to the sensory analysis, as showed by Tristezza et al [29]. Nine esters were identified and quantified, and wines produced by co-inoculation contained higher concentrations of diethyl and monoethylsuccinate, ethyl lactate, 2-phenyl acetate, and ethyl fatty acids esters [2,29].

In general, the co-inoculation of both LAB strains gave as a result a significant change in the ester profile of wine, those of ethyl fatty acid being the most representative. This suggests that coexistence of yeast and lactic acid bacteria stimulates the formation of medium chain fatty acids and, consequently, the concentration of fatty acid esters varies in wine [29,38,43]. On the other hand, the presence of 2,3-butanodiol shows that, in the case of co-inoculation, bacteria were able to degrade diacethyl, a derived-MLF compound with high impact on wine organoleptic properties [18,39]. When this compound appears in high concentrations in wine, it can affect in a negative way its aroma, conferring buttery notes that interfere with fruity aromas [44]. Consequently, applying the co-inoculation technique, it is possible to reduce unwanted buttery and lactic notes, allowing the fruity ones to prevail, bringing out the characteristic varietal aroma of Tempranillo wines. Moreover, after sensory analysis, the wines from non-inoculated LAB fermentations had the majority of high scores for these descriptors, while those fermented with LAB had the lowest scores.

It is well known that the period from the end of the AF to the beginning of the FML is specifically conducive to the development of *Brettanomyces/Dekkera*. Early wine inoculation with LAB, immediately after AF or co-inoculation, has been proved a simple and effective method to prevent *Brettanomyces* development and the production of off-flavors due to high concentrations of ethylphenols [45]. This study showed that phenol content in wine elaborated by co-inoculation of LAB starters was significantly lower than those ones by spontaneous MLF. Co-inoculation reduced the overall fermentation time by up to 2 weeks leading to a lower increase in volatile acidity and an increase of the aromatic quality of wines with more varietal and fruity notes.

Co-inoculation allows MLF to develop under reductive conditions and results in wine with very few milky and buttery flavors, related to the impact of specific compounds like 2,3-butanedione. This compound has also confirmed as being dependent on the wine bacteria used.

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